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For the President of the European Patent Office

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Yeast extracts to restore gut integrity

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YEAST EXTRACTS TO RESTORE GUT INTEGRITY

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YEAST EXTRACTS TO RESTORE GUT INTEGRITY

Field of the invention

The present invention relates to a nutritional approach having a protective effect against gut inflammation and damages.

Background of the invention

Human and animal gastrointestinal tract is at risk to develop various disorders, including these caused by aging, viruses, bacteria and/or their toxins or by physical and chemical abuses, among others.

There are several factors or therapies, which are capable of alleviating the symptoms of the various gastrointestinal disorders. Among others the indigenous flora, known as microbiota, plays an important role in modulating the intestinal environment. The non-pathogenic micro-organisms residing in the gut, known as probiotics, together with the prebiotic molecules, released from the micro-organisms or taken with the diet as food ingredients, present potential means to prevent or treat gastrointestinal disorders, including *C. difficile* infection.

It has been demonstrated that human intestinal bacteria modulate *C. difficile* toxin A production in the intestine and that toxin A binds more on intestinal membranes isolated from axenic than conventional mice, indicating that indigenous micro-organisms play an important role in *C. difficile's* pathogenesis. Clinical studies, testing nutritional approaches for treatment of *C. difficile*-induced colitis and diarrhoea, indicate that *Lactobacillus GG* improves the symptoms of colitis in hospitalised adults or infants. In a similar way the non-pathogenic yeast *Saccharomyces boulardii* has been shown to have positive effects in the prevention or treatment of *C. difficile*-induced colitis and diarrhoea in adults or infants. In addition RU 2168915 discloses the use of a meat product comprising predetermined ratios of beef, pork, blanched beef liver, squash or pumpkin, and butter as a curing or preventing food product against gastrointestinal disorders in children and weak people. All the above observations indicate that the field of nutritional intervention against *C. difficile* infection is still open.

Summary of the invention

The present invention relates to the use of an oral composition comprising yeast extracts to promote gut integrity, especially gut epithelia integrity, by preventing the disassembly of actin filaments and the disruption of tight junctions.

A second aspect of the invention relates to the use of the afore-mentionned oral composition to minimize and/or prevent gut inflammation and its consequences, in paricular those mediated by cyclooxygenase induction.

The third aspect of the invention relates to the use of the afore-mentionned oral composition to prevent the virulence and/or severity of damages caused by gastro-enteric pathogens, as enterotoxins, including those of clostridia.

Detailed description of the invention

In the present application, "oral composition" is intended to mean any ingestible composition, and may be a nutritional composition, a nutritional supplement, or a medicine.It may also be the adjuvant of a medicinal treatment, for example. It is intended to be used in humans, from infants or pre-termed infants to elderly people, having any kind of intestinal disorder. It is also intended for pets, such as cats, dogs, fishes, rabbits, mice, hamsters and the like, and more generally for any animal being bred by humans, such as horses, cows, fowl, sheep, etc, suffering from intestinal disorders.

The term "meat extract" is intended to cover extracts of any meat, such as beef, pork, sheep/lamb, chickenand/or turkey, among others. It may be an infusion of the above-cited meats, and provide at least nitrogen, amino acids, and carbon.

The term "yeast extract" is intended to cover the water-soluble portion of autolysed yeast, and preferably contains vitamin B complexes. It is also intended to cover an extract comprising both soluble and insoluble portions of autolysed bakers' yeast, and in this case it preferably further comprises riboflavin and panthotenic acid. However, in the preferred embodiment of the present invention, the "yeast extract" does not encompass the microorganism and does not comprise the enzymes produced by the microorganism. Preferably, the yeast extract is at least a yeast extract from *Saccharomyces cerevisiae*.

The term "peptone" means any enzymatic digest of any protein by the pepsin enzyme.

We have found that a selective oral delivery of food grade materials can lead to the direct correction of deleterious effects of gut upsetting, damaging or stressing agents. Therefore, the present invention relates to the use of an oral composition comprising yeast extracts to promote gut integrity.

When an intestinal disorder leads to inflammation and gut leakness or diarrhoea, it is in most cases due to the fact that gut integrity is not maintained, and more precisely that the gut's epithelium is damaged. Gut epithelium is formed by a succession of cells forming a barrier between the body and the gut lumen. Intestinal cells are linked to an other by various means, including among others tight junctions, spot desmosomes, belt desmosomes, gap junctions or infolding of membranes. Tight junctions connect two neighbouring cells thanks to transmembrane proteins that cross the cellular membranes and tight them.

Actine is also an important element of the cell cytoskeletton, allowing it to retain its structure. It can be generally considered that gut epithelia integrity is not maintained when there is a disruption of tight junctions and a disassembly of actin filaments. Disruption of epithelial cell tight junctions can be measured *in vitro* by the decrease of the transepithelial electrical resistance (TEER) of epithelial monolayers..

We have surprisingly observed that disorders affecting tight junctions of epithelial cells are counteracted when the individuals ingest yeast extracts. Preferably, yeast extracts are given in form of an oral solution comprising, in volume, from 0.01 to 5 % yeast extract. In another embodiment, the oral solution further comprises (in volume) 0.1% of meat extract and/or 1% peptone. We have demonstrated this effect both *in-vivo* and *in-vitro*.

We have also surprisingly found that individuals taking the oral composition of the present invention are less sensitive to intestinal disorders due to an impaired gut integrity and/or a damaged gut epithelia. Consequently, we do believe that the oral composition of the present invention promotes gut integrity by preventing the disruption of tight junctions and the disassembly of actin filaments.

In addition we have observed that disorders affecting the actin filaments, such as cytoskeletal alterations and subsequent cell-rounding phenomenon, are partially prevented by the ingestion of yeast extracts.

The second aspect of the invention relates to the use of the afore-mentioned oral composition to prevent the virulence and/or severity of damages caused by gastro-enteric pathogens, as enterotoxins, including those of clostridia.

Enterotoxins are toxins produced in the intestine mainly by pathogenic bacteria. Bacterial enterotoxins are potent mucosal immunogens that activate both mucosal and systemic immune responses and thus are the cause of various diseases, which include food poisoning, common diarrhoea, colitis, chronic inflammation and dysentery. Enterotoxins also lead to serious mucosal ulceration, haemorrhagic inflammatory exude or bloody diarrhoea. Toxin-induced diseases are often accompanied by abdominal cramps and rectal pain. Enterotoxins are the main stimulators of fluid secretion and intestinal inflammation. Their binding on the surface of epithelial cells leads either to desegregation of filamentous actin and to increased permeability of the tight junctions or to activation of intracellular pathways and the subsequent synthesis and release of fluid secretion activators. Toxins also induce severe inflammation, usually characterized by transmigration of neutrophils in the mucosa and enterocyte necrosis, via the activation of sensory enteric nerves and the release of sensory neuropeptides, followed by release of cytokines and epithelial cell destruction.

Examples of enterotoxin-producing bacteria are clostridia, such as *Clostridium difficile* and *Clostridium perfringens*, as well as *E. coli*, *Leishmania donovani*, *Vibrio cholera*, *Salmonella typhimurium*, *Shingellae*, *Aeromonas hydrophila*, *Staphylococcus aureus*, or *enterotoxigenic Bacteroides fragilis* (ETBF).

Clostridium difficile infection is the main cause of colitis and diarrhoea in hospitalised patients, whose intestinal microbiota is altered due to antibiotics uptake. *C. difficile* causes enteritis by releasing two enterotoxins: toxin A and toxin B. Both toxins have a potent cytotoxic effect in humans but toxin A is the main stimulator of fluid secretion and intestinal inflammation. Toxin A binds on the surface of epithelial cells and it is internalised into the cytoplasm in coated pits. Internalisation leads to disassembly of actin stress fibers, disruption of the actin-associated adhesion plaque, opening of the tight junctions, cell detachment and increased fluid secretion. Those effects have been demonstrated *in vitro* on cultured human epithelial cell lines, such as the T84 colonic cell line, where addition of toxin A on the monolayer diminished the transepithelial resistance and increased the permeability of the monolayer. *C. difficile* enterotoxins *in vivo* have been shown to induce severe inflammation, characterized by transmigration of neutrophils in the mucosa and enterocyte necrosis, when guinea pig, rabbit or rat

ileum have been exposed to toxin A. The mechanism leading to this acute inflammatory response appears to be the activation of sensory enteric nerves and the release of sensory neuropeptides. Recent studies also proposed that toxin A upregulates expression of COX-2 in the intestine.

One of the most common consequence of damages caused by gastro-enteric pathogens is diarrhoea. Diarrhoea is the result of increased secretions from the epithelial cells in the gut, which may be induced by parasites, pathogenic bacteria (including enterotoxin-producing bacteria), or viruses.

An aspect of the present invention lies in the following fact: when an individual ingests an oral composition comprising yeast extracts, said ingested composition inhibits the intestinal fluid secretion causing diarrhoea.

A third aspect of the invention relates to the use of the afore-mentionned oral composition to minimize and/or prevent gut inflammation and its consequences, in paricular those mediated by cyclooxygenase (COX-2) induction.

COX-2 is an enzyme catalyzing the synthesis of prostaglandins from arachidonic acid. Other known substrates for COX-2 include dihomo-gamma-linolenic acid (20:3n:6) and eicosapentaenoic acid (EPA, 20:5n-3) producing PGE_1 and PGE_3, respectively. The human COX-2 gene has been cloned and its genomic pattern and the responsiveness of its gene expression to different elements, such as cAMP, NF-κB and TGF-ß, IL-1 or TNF-α has been described.

COX-2 is linked to numerous inflammations, including allergic reactions and gut inflammations. Among gut inflammations and disorders wherein COX-2 activity is involved are gastritis, inflammatory bowel disease, irritable bowel syndrome, or intestinal cancers.

We have found that the COX-2 pathway is involved in the protective action of yeast extracts.

The composition of the invention, that is to say comprising yeast extracts, has been found to preserve the gut barrier and the gut integrity, thus being very intersting in numerous aspects. One of the populations that could benefit from the present invention is the infant population; gut integrity is often challenged in infancy. The present invention, properly used, prevents gut leakiness and diarrhoea, e.g. provoked

by infectious agents such as, bacteria or viruses. Another target population consists of hospitalised patients: the present invention protects them from nosocomial infections, and helps the management of their gut inflammation. Furthermore, the composition of the invention is usefull for reparing gut functions impaired in aging individuals and in inflammation situations, as well as for reparing gut functions impaired from unusual diets, such as ethanol and/or drug consumptions, for example.

The composition of the invention can be incorporated to a number of food products. For example, it can be incorporated to infant formula powder when the target population is an infant population; it can also be incorporated to dehydrated food products, such as soups. It can further be incorporated in canned oral supplement formulas. Example of such canned formulas are these intended for the enteral nutrition of individuals.

When the individual suffering from an intestinal disorder is a pet, the composition according to the invention can be any wet or dry pet food.

When the composition according to the invention is incorporated into a medicine, it can be incorporated together with any appropriate excipient to any medicinal form.

We have found that by ingesting yeast extracts, individuals having intestinal disorders have a normalised fluid secretion, a cellular structure less damaged, and a decreased inflammation compared to individuals having the same disorders but a diet not supplemented with yeast extracts.

In the frame of the present invention, yeast extracts may also be associated with meat extracts, peptones, or a combination of both to obtain an improved effect on gut integrity into individuals subjected to gut upsets, damages and stresses.

Examples

The following examples are illustrative of some of the products falling within the scope of the present invention and methods of making the same. They are not to be considered in any way limitative of the invention. Changes and modifications can be made with respect to the invention. That is, the skilled person will recognise many variations in these examples to cover a wide range of formulas, ingredients, processing, and mixtures to rationally adjust the naturally occurring levels of the compounds of the invention for a variety of applications.

Example 1 – effect of the composition on tight junctions and actin filaments

Material and methods

The human colonic cell line T84 (ATCC, CCL-248) was cultured in DMEM:F12 1:1 supplemented with 20% FBS (Foetale Bovine Serum) 2 mM glutamine and 100 U/ml penicillin-streptomycin. Human primary skin fibroblasts were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin.

T84 monolayers were seeded in 6-well inserts plates at $0.5x10^6$ cells/insert and cultured during 3 weeks. The basal value of the TEER (transepithelial Electrical resistance) was measured and culture medium was replaced by 20% a combination of meat extracts, peptone and yeast in PBS or 20% of a solution containing 0.5% yeast extract or 1% beef extract or 1% peptone. After 1 h at 37°C, *C. difficile* toxin A was added in the apical side of the monolayers at a final concentration of 100 ng/ml and the TEER were further measured after 1, 2, 4, 6 and 24 h at 37°C. Control monolayers were exposed to cultured media only. For each condition triplicate inserts were used. At each time point, 1 ml apical and 1 ml basolateral medium was collected and cell viability was evaluated by measuring the LDH release using the Cytotoxicity Detection Kit according to the manufacturers' instructions.

T84 cells or human primary fibroblasts ($2x10^5$/chamber) were seeded on 4-chamber glass slides, grown as described previously and incubated with a combination of meat extracts and peptone (hereinafter "the composition"), yeast extract, beef extract or peptone solutions for 1 h before addition of toxin A at a final concentration of 500 ng/ml. After 6 h, cells were washed with PBS, fixed with 3.7% paraformaldehyde, washed twice with PBS, permeabilized for 5 min at –20°C with acetone and treated with PBS-1% BSA (Bovine Serum Albumin) to reduce non-specific labelling. Actin desegregation and cell rounding were assessed by fluorescent microscopy after labelling with 200 U/ml rhodamine-labelled phallotoxin.

Results

Toxin A affects tight junctions of epithelial cells, an effect which is measured by the decrease of the transepithelial electrical resistance (TEER) of epithelial monolayers. To assess whether the composition could counteract the virulence of toxin A, T84 monolayers were exposed to toxin A in the presence or absence of the composition and TEER were measured. Addition of 100 ng/ml toxin A to T84 monolayers resulted in a 3-fold reduction of TEER control values after 6 h of incubation (309±8 vs.

985±49 Ωcm^2). Addition of 20% the composition together with toxin A, prevented the TEER decrease (1403 ±95 vs. 309±8 Ωcm^2), while it did not alter the basal TEER values of T84 cells (1217±277 Ωcm^2 vs. 985±49 Ωcm^2). No modifications in cell viability were observed, indicating that toxin A does not induce cell death during the 6 h period. The above results demonstrate that the composition could counteract toxin A and protect T84 monolayers from toxin A-induced TEER decrease.

To determine whether the protective effect of the composition against toxin A-induced TEER decrease was correlated with alteration of the cytoskeleton leading to cell rounding, T84 cells were treated with toxin A alone or in combination with 20% of the composition and cytoskeletal actin was analysed by immunocytochemistry. Addition of 500 ng/ml toxin A induced T84 cell rounding, which is evidenced by the bee nest appearance of the cell monolayer due to actin desegregation, and packaging. Addition of 20% of the composition in combination with toxin A partially prevented actin desegregation and subsequent cell rounding induced by toxin A, while it did not influence the cytoskeleton of the cells when added alone. These effects were hardly visible due to the spatial configuration of the T84 monolayer. To render the interpretation easier, experiments were repeated using primary human skin fibroblasts, which form a planar monolayer. After 6 h in the presence of toxin A, all fibroblasts presented a round appearance indicaitng a complete cytoskeleton disruption. Addition of 20% of the composition in combination with toxin A partially prevented actin desegregation and cell rounding. The shape of fibroblasts treated with toxin A and 20% of the composition was comparable but not identical to the shape of control fibroblasts or of fibroblasts treated with the combination alone. Thus the composition could counteract toxin A, partially preventing cytoskeletal alterations and subsequent cell rounding, due to actin desegregation.

Discussion

The mechanisms of the protective action observed here are not clearly elucidated and probably are diverse. Toxin A induces polymerisation of actin filaments, leading to desegregation of cytoskeletal actin. Actin disruption is the cause of cell rounding, observed *in vitro*, and increased permeability of the tight junctions. The toxin A effect on actin is due to its glucotransferase activity against the Rho family of proteins. Toxin A is able to enzymatically transfer a glucosyl residue from UDP glucose to threonine 37 of Rho, Rac and Cdc-42, leading to disassembly of actin stress fibers, disruption of the actin-associated adhesion plaque, opening of the tight junctions, cell detachment and increased fluid secretion. Those effects have been demonstrated *in vitro* on T84 cells, where addition of toxin A on the monolayer diminished the

transepithelial resistance and increased the permeability of the monolayer, due to modifications of the Rho proteins in the epithelial cells. Therefore we believe that peptone, yeast extract, and beef extract interfere with the signalling pathway of the Rho proteins, inhibiting the effects of toxin A. Although not wishing to be bound by theory, this interference could be up-stream or down-stream of the transfer of the glucosyl residue to Rho proteins.

Example 2 - effect of the composition on damages caused by enterotoxin-producing gastro-enteric pathogens

Material and methods

Six weeks old male mice were treated *ad libitum* with 60 mg/L gentamicin, 250 mg/L vancomycin, 300 mg/L amoxicillin and 10 mg/L amphotericin for a week in order to eliminate the intestinal microbiota. Mice were then divided into three groups: I) a control group; ii) a group receiving *ad libitum* 20% of the composition or 20% of a solution containing 0.5% yeast extract or 1% beef extract or 1% peptone in the drinking water, for a week; and iii) a group that was gavaged twice with 500 µl the composition or the composition components at two day interval. The day after the end of treatments, animals were anaesthetised with 30 mg/kg of body weight sodium pentobarbital (?) and placed on a warm blanket (37° C), under 0.8-3% isofluoran anaesthesia for the whole duration of the operation. The abdomen was opened by a midline incision and the distal jejunum was exposed. Two 5 cm jejunal segments were doubly ligated at each end with surgical thread to form two intestinal loops with a 2 cm interval between them. One loop was injected with 600 µl PBS as a control and the other with 600 µl PBS containing 20 µg toxin A. The intestinal loop was then returned to the abdominal cavity and the incision was sutured closed. Mice were allowed to recover and they were followed continously. Animals were euthanised after 4 h, the loops were isolated and their weight to length ratio (in mg/cm) was recorded to estimate fluid secretion. Loops were then washed twice with ice cold PBS, dipped in RNA*later*™, flashed frozen in liquid nitrogen and stored at -80° C.

Results

C. difficile infection, leading to diarrhoea and colitis, develops mostly in hospitals and elderly people's home striking patients who take antibiotics and thus their intestinal microbiota is altered. To assess whether the composition of the invention and its components can counteract toxin A effects *in vivo*, a mouse model was used. To mimic the conditions that trigger *C. difficile* infection in humans, mice were treated for a week with antibiotics aimed to alter their intestinal microbiota. One day after the

end of antibiotic treatment, a group of mice were given 20% of the composition *ad libitum* for one week. At the end of this period, intestinal loops were formed and injected with PBS or 20 µg toxin A. After 4 h incubation, loops from control mice exhibited an increased fluid secretion when injected with toxin A compared to PBS injected loops (121.9± 31.7 vs. 64.6± 13.5 mg/cm). In contrast, in mice receiving the composition for one week, no differences in fluid secretion were observed in loops injected with toxin A or PBS (73.6±8.3 vs. 66.8±10.8 mg/cm). Similar results were obtained when mice were given by gavage two doses of 500 µl the composition. These results show that treatment with the composition can prevent the adverse effect of toxin A in subjects exhibiting an impairment of the intestinal microbiota.

To determine whether the composition exerts its protective action by direct inactivation of toxin A, 20% of the composition or PBS as a controlwere mixed with toxin A 1 h before injecting the mixture in the intestinal loops of mice, treated for a week with antibiotics. There was not a significant difference recorded between control (PBS) and the composition-injected loops at the level of toxin A-induced fluid secretion. This result indicates that the composition does not counteract the effects of toxin A via direct binding and inactivation of the toxin, which could lead to either toxin-cleavage or masking of the toxin-binding epitopes.

Discussion

Peptone, yeast extract and beef extract protect mice from intestinal fluid secretion induced by toxin A. Although not wishing to be bound by theory, we believe that the protective action of peptone, beef extract and yeast extract are not due to an enzymatic activity, which cleaves toxin A for two main reasons: i) The solutions used were always autoclaved, which would lead to disactivation of any enzymes, such as proteases, contained in the solution and ii) The composition mixed and incubated with toxin A before being injected in the intestinal loops of mice, could not inhibit intestinal fluid secretion. Therefore we do believe that the protective activity of peptone, yeast extract and beef extract is due to the presence of free molecules in the solution (e.g. aminoacids or peptides), which could bind on the toxin A receptor on the intestinal epithelial cells and prevent binding of toxin A and the activation of the signalling pathways involved.

Example 3 - effect of the composition on the expression of COX-2

Materials and methods

The same procedure described in example 2 was used. The RNA was extracted from mouse intestinal loops, and COX-2 mRNA expression was assessed by RT-PCR.

Total RNA (1 µg) was reverse transcribed with 200 U of Superscript II® enzyme. A 400 bp fragment of mouse COX-2 was amplified by PCR using 5'-CACAGTACACTACATCCTGACC-3' as sense and 5'-TCCTCGCTTCTGATTCTGTCTTG-3' as antisense primers.. A 700 bp fragment of β-actin, used as an internal control, was amplified from the same RT mix with the 5'-ATGAGGTAGTCTGTCAGGT-3' as sense and 5'-ATGGATGACGATATCGCT-3' as antisense primers. To exclude DNA contamination, PCR was performed directly on RNA samples. PCR products were loaded on 1% agarose gel, photographed, and pictures used for densitometrical quantification of band intensities. Normalization was performed against the expression of the internal control β-actin: the rations of the COX-2 and the corresponding β-actin mRNA signals were determined and expressed relative to that of the "not-treated sample" (given water and injected with PBS) to which an arbitrary score of 1 was assigned.

Results

To determine whether COX-2, known to be involved in toxin A-mediated fluid secretion, is also implicated in the composition's protective effect, COX-2 mRNA expression was assessed by RT-PCR. Changes in COX-2 expression due to different treatments were expressed relative to β-actin. Injection of 20 µg toxin A in the intestinal loops of control mice resulted in a 3.6-fold increase of COX-2 mRNA expression. Treatment of mice for one week with the composition resulted in a 2-fold reduction of the COX-2 increase mediated by toxin A. Intestinal COX-2 expression induced by toxin A was completely normalised to basal levels in mice under peptone or beef extract treatments while it was decreased by 2.3-fold under yeast extract treatment. Neither the composition nor its components significantly modified the basal levels of COX-2 mRNA expression. When given by gavage, the composition or its components were also able to counteract the increase in COX-2 mRNA induced by toxin A.

Discussion

When toxin A binds on the epithelial cells it is shown to induce inflammation, including neutrophil migration and enterocyte necrosis and destruction of the villus. Those effects are mediated by the release of sensory neuropeptides, such as substance P and calcitonin gene-related peptide, following the activation of sensory enteric nerves. In addition expression on the intestinal epithelium of NK-1R the receptor for SP significantly increases both in animals and in humans infected with *C. difficile*. Recent studies also proposed that toxin A of *C. difficile* upregulates expression of COX-2 in the intestine. COX-2 is the inducible isoform of the cyclooxygenase

enzyme, which mediates synthesis of prostaglandin E2 (PGE2) an agent known to increase intestinal fluid secretion, which leads to diarrhoea. Although not wishing to be bound by theory, we believe that peptone, yeast extract and beef extract inhibit any of these pathways counteracting toxin A. Our results indicate that peptone, yeast extract or beef extract inhibit intestinal, toxin-mediated , COX-2 induction. This could be due to inhibition of toxin A-mediated signalling, which leads to COX-2 activation if our solutions inhibit or reduce binding of the toxin on its intestinal receptor.

Abstract

The present invention relates to the use of an oral composition comprising yeast extracts to promote gut integrity, especially gut epithelia integrity, by preventing the disassembly of actin filaments and the disruption of tight junctions.

A second aspect of the invention relates to the use of the afore-mentionned oral composition to minimize and/or prevent gut inflammation and its consequences, in paricular those mediated by cyclooxygenase induction.

The third aspect of the invention relates to the use of the afore-mentionned oral composition to prevent the virulence and/or severity of damages caused by gastro-enteric pathogens, as enterotoxins, including those of clostridia.